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EVALUATION OF PRESERVATIVE PROPERTIES AND ANTIMICROBIAL ACTIVITIES OF *Anogeissus leiocarpus* EXTRACT ON FOOD PATHOGENS OF *Hibiscus sabdariffa* CALYX (ZOBO) DRINK

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ABSTRACT
The study was aimed at evaluating phytochemical constituents, antimicrobial and preservative activities of A. leiocarpus extract on zobo drink. The plant materials were sourced, identified and extracted using water and ethanol. Preliminary phytochemical screening of extracts and fractions was carried out using standard procedure. Isolation, identification of bacterial and fungal species commonly implicated in food borne illness and spoilage were carried out using standard protocol. Evaluation of antimicrobial and preservative activities of the extracts and fractions was also carried out. Result of phytochemical screening revealed the presence of saponnins, anthraquinones, alkaloids and tannins in aqueous and ethanol extracts of A. leiocarpus. Alkaloids and anthraquinones are present in all the fractions. The antimicrobial activities result showed ethanol extracts of A. leiocarpus possessed better antimicrobial activity among the extracts tested with zone diameter of 24.0±2.00mm at 2000µg/ml concentration against S. aureus. The activity of ethanol extract of A. leiocarpus at 2000µg/ml against E. coli, Salmonella spp and Shigella spp was 20.0±0.0mm, 13.5±0.50mm and 20.5±0.50mm. Antimicrobial activity of acetone fraction showed E. coli is sensitive (19.0±0.00mm) at 2000µg/ml but no significant difference (p<0.05) was observed when compared with other organisms. Treatments A, B, C and D showed significant decrease in Aerobic Bacteria Count by 0.62 log, 0.16 log, 0.77 log and 0.35 log after 48hrs of storage. Conclusively A. leiocarpus aqueous and ethanol extracts possess antimicrobial and preservative activities which should further be evaluated against different food systems.
Keywords: Phytochemicals, Antimicrobials, Fraction, Spoilage, Preservatives

INTRODUCTION

The antimicrobial activity of plants extracts has formed the basis of many applications including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (El Ghazali *et al.*, 2003*)*.). It has been estimated that 25% of the modern medicine are made from plants first used traditionally. Several researches have been conducted on the medicinal potential of *Anogeissus leiocarpus* for treatment of cough (El-Ghazali *et al.*, 2003), malaria, trypanasomiasis, helminthiasis and dysenteric syndrome (El Ghazali *et al.*, 2003*)*.

Active antimicrobial compounds of *A. leiocarpus* are tannins, flavonoids, terpens, saponins, and alkaloids (El Ghazali *et al.*, 2003*)*.

Anogeissus leiocarpus is a deciduous tree species that can grow up to 15–18 m of height and measure up to 1 m diameter. Bark grayish, scaly. Branches often drooping and slender, leaves alternate, ovate lance late in shape, 2-8 cm long and 1.3-5 cm across. The leaves are acute at the apex and attenuate at the base, pubescent beneath. Inflorescence globes heads, 2cm across, yellow; the flowers are bisexual, petals absent. Fruits are globes cone like heads; each fruit is broadly winged, dark grey, 3cmacross. It can reproduce by seeds as well as vegetative propagation (El Ghazali *et al.*, 2003*)*.Many traditional uses have been reported for the plant. In Sudanese traditional medicine the decoction of the barks is used against cough. Rural populations of Nigeria use sticks for or dental hygiene, the end of the sticks are chewed into fibrous brush which is rubbed against teeth and gum. Ivory Coast traditional practitioners use the plant for parasitic disease such as Malaria, Trypansomiasis, Helminthasis and dysenteric syndrome In Togolese traditional medicine It is used against fungal infections such as dermatitis and Mycosis, also the decoction of leaves is used against stomach infections. It is in view of the numerous benefits of the plant mentioned above that the aim of this research was set up to evaluate its antimicrobial and preservative effect on *Zobo* drink.

MATERIALS AND METHODS

Collection, authentication and processing of plant materials

The stem bark of *A. leiocarpus* were collected from Sabongarin Takanebu, Miga Local Government Area, Jigawa State, Nigeria. The plant material were identified and authenticated by a Botanist at the Plant Biology Department, Bayero University, Kano, Confirmation of taxonomic identity of the plant was achieved by comparison with voucher specimens kept at the Herbarium of the Department of Plant Biology, BUK and use of documented literature from Dalziel (1916). Accession number was assign to the plant as BUKHAN 0029. The plant materials were air-dried in the laboratory for four weeks and then ground into powdered form, using a mortar and pestle, and stored for future use.

Extraction of Plant Material

The powdered plant material (100g) of stem bark were percolated in 1000mL ethanol in separate 2L capacity conical flasks, stoppered and kept for two weeks with intermittent shaking. The percolates were filtered with Whatman's No 1 filter paper. The extract were concentrated at students water bath in the post graduate laboratory Bayero University, Kano. The same quantity of plant material was again percolated with distilled water for one week and after filteration, the aqueous extract was concentrated in hot oven at 40^{o}C (Fatope *et al.,* 1993).The pure extract were transfer into airtight container and store at 40^{o}C prior to use.

Phytochemical Analysis of plant extracts

Phytochemical analysis for qualitative detection of alkaloids, flavonoids, tannins, anthraquines and saponins was performed on the extracts as described by Trease and Evans (1989), Sofowora (1982).

Sources of Microorganisms

*Staphylococcus aureus, Salmonella s*pp, *Shigella* spp*, E. coli, Pseudomonas aeruginosa, Aspergillus Spp, Paecilomyces spp, Penicillium spp and Nigrospora spp* were isolated from zobo drink. The zobo drink were homogenized and streaked on appropriate media for isolation, cultural, morphological and biochemical characterization of isolates were carried out using procedure described by Cheesbrough (2004).

Preparation of Concentration of the Extracts

Stock solution (20mg/ml) of the plant extract was prepared by dissolving 0.2g of the plant extract in 10ml of DMSO. Four concentrations were prepared from the stock solution using serial doubling dilution such that 0.1ml was placed in each well equivalent to 250µg/ml, 500µg/ml, 1000 µg/ml and 2000 µg/ml respectively. The assessment of antibacterial activity was based on measurement of the diameter of zone of inhibition formed around the well.

Standardization of Innoculum

Inoculum where prepared from the cultures maintained on a slant of nutrient agar (Bacteria) and potato dextrose agar (PDA) for fungal isolates. Density of bacterial suspension was adjusted to 0.5 McFarland standard (Barium sulphate solution) (1.5 x 10^{8}cfu/ml) as described by Cheesebrough (2002). Aloopful of fungal spores from an overgrown plate was taken and shaken thoroughly in 10ml of 20% Tween 80 solution to arrive at 6.0×10^{5}cfu/ml of the spore suspension (Murugan *et al.,* 2007).

Susceptibility Testing

Agar diffusion technique described by (Bauer *et al.,* 2008) was employed for antibacterial and antifungal bioassay. The assessment of antibacterial and antifungal activity was based on measurement of the diameter of the inhibition zone formed around the well.

Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of the active concentrations were determined using the tube dilution technique described by (Lima *et al.*, 1993) Appropriate test concentrations were prepared and added to sterile capped test tubes of Mueller Hinton Broth (Bacteria) and Potato Dextrose Agar (fungal isolates) to cover the range of dilutions chosen in duplicate After overnight incubation at 37^{o}C, the lowest concentration of the extract at which no turbidity was observed and recorded as the Minimum Inhibitory Concentration

Determination of Minimum Bactericidal (MBC) and Fungicidal Concentration (MFC)

Sterile Mueller-Hinton agar plates were inoculated with samples from the MIC tubes that show no visible bacterial growth. The lowest concentration in which no growth occurred on the medium was considered as the MBC (Lima *et al.*, 1993).

Gas Chromatography-Mass Spectrophotometry (GC-MS) analysis

GC-MS analysis was conducted on the most active fraction of *A. leiocarpus* from the bioactivity guided fractionation to determine the molecular weight, IUPAC name and chemical structures of some of the functional group that were identified during the analysis.

Sanitization of 'Zobo Drink'

A set of 16 20 ml capacity sterile bottles were filled with 10ml of the zobo drink each. Four different treatments were carried out as follows:

A – Untreated 'Zobo' with no additive.
B - Treatment with 0.5% w/v of Extracts
C – Treatment with 1.0% w/v of Extracts
D - Treatment with 0.5% w/v sodium benzoate (conventional preservative for drinks).

Enumeration of Aerobic mesophilic bacteria and fungi was carried out in triplicate determinations. The mean value was taken, and the room temperature, pH was monitored throughout the period of experiment as described by Bukar (2012).

Sensory Analysis

Organoleptic parameter (colour, odour and texture) were assessed by a panel of judges through sensory evaluation (Idise, 2011).The scores were graded on hedonic scale as 9 (like extremely) being the highest value and one(1) being the lowest value (dislike extremely) and after a period of storage ,the treatment most preferred by the judges and the number of days for each treatment to deteriorate were recorded and compared with the control (Bukar ,2012).

Statistical Analysis

Data generated from the sanitizing activity tests and the scores generated based on the assessment of judges (from the preservative experiments) using Hedonic scale, were statistically analyzed using Analysis of Variance (ANOVA) at 0.05 probability level using the software package developed by Microsoft corporation. Where significant difference was observed, the means separation was carried out using least significant difference (LSD).

RESULTS AND DISCUSSION

Table 1 shows the physical characteristics of the extracts the ethanol recovered more of the extract as compare to water. More polar solvents (such as water and ethanol) recovered more extracts than less polar solvents such as chloroform. The results from table 1 revealed the taste of the various extracts which is very important revelation as the extracts would be use in sanitizing and preservative experiments. The strength of their taste might interfere on the sensory properties of the foods, which could determine their acceptability or otherwise. The results of phytochemical analysis showed the extracts to revealed the present of secondary metabolites such as saponins, anthraquinones, alkaloids and tannins in ethanol extract. Virtually all the screened secondary metabolites are detected in the aqueous extract.(Table 2). This corroborated the work of Bukar (2012) who equally detected saponins, tannins and flavonoids in aqueous extract of stem bark of *A. leiocarpus* , This is also in line with report of Kubmarowa (2007) who reported the present of alkaloids and flavonoids in the two extracts. Abdu and john bull (2006) also reported the presence of tannins, saponnins and flavonoids in ethanol extract of stem bark of *A. leiocarpus*.

Table 1: Physical Characteristics of the Extracts

Physical Parameters	EESBAL	AESBAL
Weight of extract/leaves (g)	100	100
Weight/ volume extracted	14.8g	13.9g
% extract	14.8	13.1
Color of extract	Coffee colour	Honey colour
Texture	Gummy	gummy

Key; EESBAL=Ethanol extract of stem bark of *A. leiocarpus*, AESBAL= Aqueous extract of stem bark of *A. leiocarpus*.

Table 2: Phytochemical constituents of extracts of *A. leiocarpus*

	EESBAL	AESBAL
Saponnins	+	+
Anthraquines	+	+
Alkaloids	+	+
Tannins	+	+
Flavonoids	-	-

Key; EESBAL=Ethanol extract of stem bark of *A.leiocarpus,* AESBAL= Aqueous extract of stem bark of *A.leiocarpus*, + = Presence, - = Absence.

In-vitro antimicrobial activities indicated that the extracts were active against the test organisms as presented in Table 3. *E. coli, S.aureus* and *Shigella* spp were sensitive with zone diameter of 20.0mm,24.0mm and 20.5mm respectively, *Salmonella* spp has the least sensitivity of 13.5mm while *Nigrospora* spp, *Penicillium* spp, *Aspergillus* spp and *Paecilomyces* spp were sensitive to ethanol extract with zone diameter of 17.0mm,16.0mm and 15.0mm respectively.

All the tested organisms showed less active against the aqueous extract Table 3. From the result, it could be observed that ethanol extract had the broadest spectrum of activity on the test organisms This findings agrees with research findings of Bukar (2012) who reported the antibacterial activity of ethanol extract of stem bark of *A .leiocarpus* against *E. coli*, *Shigella* Spp, *Salmonell*a Spp and *S. aureus*. It also corroborate the work of Abdu and Johnbull (2006) who reported on the antibacterial activity of ethanol extract on *S.aureus* and *P.aeruginosa*..The antifungal activity of the extract is due to their ability to prevent spore germination by breaking down the spore wall on contact with the extracts (Rana *et al.*, 1997)

Table 3: Zone diameter of inhibition (mm) of A. leiocarpus extracts on bacterial and fungal isolates

	EESBAL				*AESBAL*			
Organisms	250	500	1000	2000	250	500	1000	2000
E.coli	7.5±0.5	15.5±1.5	17.0± 0.0	20.0±0.0	0.0±0.00	6.5±0.50	8.5±0.50	9.5±0.50
P.aeruginosa.	7.5±0.5	11.0± 1.0	12.0± 0.0	15.0± 0.0	0.0±0.00	0.0±0.00	9.0±1.0	14.0±1.0
S.aureus	10.5±0.5	14.5±0.5	19.5±0.5	24.0± 1.2	0.0±0.00	0.0±0.00	9.5±0.5	12.5±0.5
Salmonella spp	8.5±0.5	10.0±0.0	11.5± 0.5	13.5± 0.5	2.5±2.5	10.0±0.0	12.5±0.5	13.5±0.5
Shigella spp	10.5±0.5	13.5±0.5	15.5±0.5	20.5± 0.5	8.0±0.0	10.5±0.5	13.0. ±1.0	14.5±0.5
Aspergillus spp	9.5±0.5	12.5± 0.0	13.5±10.0	15.0± 1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Nigrospora spp	10.0±1.0	12.5±0.5	15.5±0.0	17.0±1.0	0.0±0.0	6.5±0.5	8.5±0.5	10.0±0.0
Paecilomyces. spp	9.0± 0.0	11.0± 1.0	12.5± 2.5	15.0±1.0	14.0±0.0	0.0±1.0	0.0±0.0	0.0±0.0
Penicillium spp	9.5±0.5	12.0±0.0	14.5±0.0	16.0±0.0	0.0±0.0	6.0±0.0	8.0±0.0	10.0±0.0

Key; EESBAL=Ethanol extract of stem bark of *A. leiocarpus*, ,AESBAL= Aqueous extract of stem bark of *A. leiocarpus* Values are mean ± SE and each value is mean of three determinations 6mm;size of the well.

Table 4 show minimum inhibitory concentration of stem bark of *A. leiocarpus*, is the lowest concentration that inhibits the growth of microorganism from the table could be observed that MIC on AESBAL against *E. coli* and *S. aureus* at 500µg/ml and 250 µg/ml showed growth not inhibited but wit growth inhibition at 500 against *P. aeruginosa*. Table 5 showing the result of Minimum bactericidal concentration of aqueous and ethanol extract of *A. leiocarpus*, MBC of EESBAL on *E. coli*, *S. aureus, Shigella* spp and *Salmonella* Spp at 500 µg/ml, 250 µg/ml, 125 µg/ml and 62.5 µg/ml inhibit the growth of these microorganisms but *P. aeruginosa* growth are not inhibited at 500 µg/ml and 250 µg/ml concentration.

Table 4: Minimum inhibitory concentration of stem bark extract of *A. leiocarpus*

Diameter of zone of inhibition (mm))/ extract concentration (µl/ml

	EESBAL MIC				**AESBAL MIC**			
	500	**250**	**125**	**62.5**	**500**	**250**	**125**	**62.5**
E. coli	>500	>250	>125	>62.5	500	>250	>125	>62.5
S. aureus	>500	>250	>125	>62.5	500	250	>125	>62.5
Shigella spp	500	>250	>125	>62.5	500	250	>125	>62.5
Salmonella spp	>500	>250	>125	>62.5	500	250	>125	>62.5
Pseudomonas aeroginosa	500	250	>125	>62.5	500	>250	>125	>62.5

Key: EESBAL: Ethanolic extract of stem bark of *A. leiocarpus*.
AESBAL: Aureus extract of stem bark of *A. leiocarpus*
>concentration : growth not inhibited, Exact concentration : growth inhibited

Table 5 :Minimum Bactericidal Concentration of stem bark of *A. leiocarpus*

Diameter of zone of inhibition (mm))/ extract concentration (µl/ml

	EESBAL				**AESBAL**			
	500	**250**	**125**	**62.5**	**500**	**250**	**125**	**62.5**
E. coli	-	-	-	-	500	-	-	-
S. aureus	-	-	-	-	500	250	-	-
Shigella	-	--	-	-	>500	>250	-	-
Salmonella	-	-	-	-	>500	>250	-	-
Pseudomonas	>500	>250		-	>500	-	-	-

Key: EESBAL: Ethanolic extract of stem bark of *A. leiocarpus*.
AESBAL: Aqueous extract of stem bark of *A. leiocarpus*
>concentration : growth not inhibited, Exact Concentration; growth inhibited.
-; already growth not inhibited in the MIC

Functional group identified from the results of the Gas chromatography-mass spectrometry (GC-MS) analysis were found to be hydrocarbons, which were either acyclic, alicyclic (monocyclic, bicyclic, or tricyclic), or aromatic.

Aromatic compounds like the cyclohexene characterized by sharp smell, and unstable compound which is a precursor of Maleic acid and Adipic acids .Some of the functional group include Pelargonic alcohol, trans-3-Undecene n-Pentadecanoic acid Methyl-.beta.-D-arabinopyranoside and alpha.-L-Galactopyranoside, methyl 6-deoxy,3H-Pyrazol-3-one, 2,4-dihydro-4,4,5-trimethyl, n4-Hexen-3-one, 4,5-dimethyl, Borane, isopropyldipropyl and Octadecanoic (Table 6).

Table 6: GC-MS analysis for the chloroform fraction of *A. leiocarpus* ethanol Extract

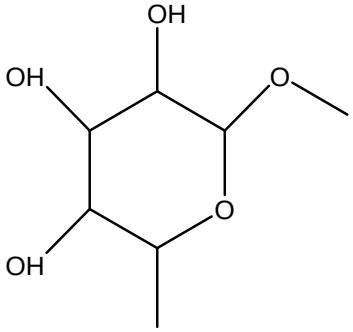
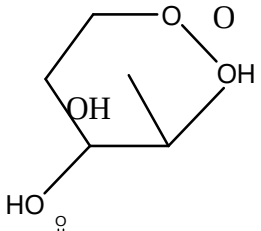
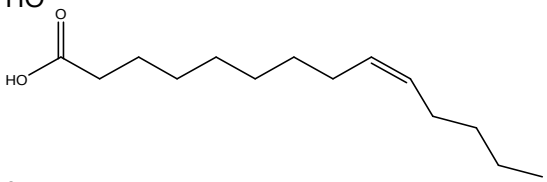
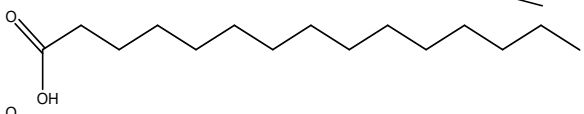
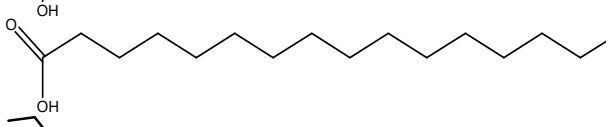
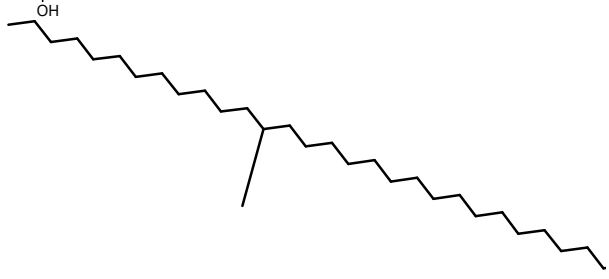
S/N	Molecular weight	IUPAC Name	Molecular formula	Chemical structure
1.	178	alpha.-L-Galactopyranoside, methyl 6-deoxy	C7H14O5	
2.	242	Methyl-.beta.-D-arabinopyranosid	C6H12O5	
3.	254	Hexadecenoic acid	$C_{16}H_{30}O_2$	
4.	242	Pentadecanoic acid	$C_{15}H_{30}O_2$	
5.	298	Nonadecanoic acid	$C_{19}H_{38}O_2$	
6.	508	Heptadecanoic acid, heptadecyl ester	C34H68O2	

Table 7 showed the temperature and pH of treated and untreated zobo drink for 96hrs of storage. pH of the untreated zobo (A) at 0hr to 96hrs are 4.08,4.2,4.28,4.19 and 4.18 respectively. There is increase in pH from 0hr to 96hrs i.e 4.08 to 4.18. Treated zobo with 0.5% extract (B) had a pH of 4.19 at 0hr and 4.15 at 96hrs there is decrease in the pH. Zobo treated with 1% (C) had a pH of 4.19 at 0hr and 4.16 at 96hrs there is also decrease in pH. Zobo treated with 1% sodium benzoate had a pH of 6.09 at 0hr and 6.14 at 96hrs there is increase in the pH. Temperature of untreated and treated zobo drink at 0hr, 24hrs, 72hrs and 96hrs are 32^0C, 32^0C, 30^0C, 32^0C and 32^0C respectively. Generally the temperature remains the same but with little changes throughout the experiments. Therefore the results showed that temperature and pH vary slightly, though not significantly in line with report by Doughari (2007) who equally reported slight variation in pH during storage of zobo drink for fourteen days. This is also in line with work of Bukar (2012) who equally reported on the variation of temperature and pH of zobo drink.

Table 7: Temperature and pH of treated and treated zobo after 96hrs of storage

		Treatments			
Parameters	Time (Hr)	A	B	C	D
Temperature (^{o}C)	0	32	32	32	32
	24	32	32	32	32
	48	30	32	32	34
	72	32	32	32	32
	96	32	32	32	32
pH	0	4.08	4.08	4.08	4.08
	24	4.2	4.19	4.19	6.09
	48	4.28	4.19	4.16	6.05
	72	4.19	4.19	4.20	6.12
	96	4.18	4.15	4.16	6.14

The result from the sanitizing activity of *A. leiocarpus* ethanol extract on prepared zobo drink is presented in Figures 1 and 2. It could be observed that from 0 to 24 hours, all the treatments had bacteria count reduction with treatments C and D having the highest count reduction of 0.81 and 1.21*log* cfu/ml with the least being treatment B with 0.17*log* cfu/ml. Thereafter, there was log count increase for up to 72 hours (Figure 1).

Fungal count decreased in treatments B, C and D from 0 to 72 hours, with treatments C and D having the highest *log* count decrease of 0.22 and 0.13 *log* cfu/ml respectively. Thereafter, from 72 to 96 hours there was *log* count increase in all the treatments (Figure 2).

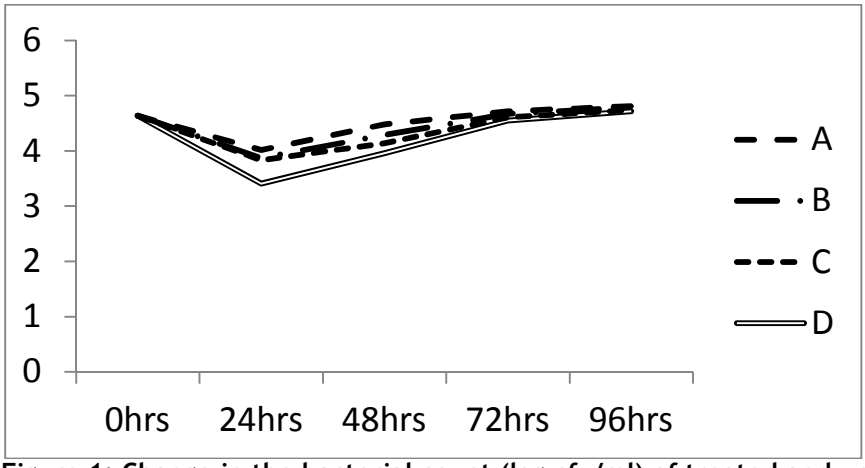


Figure 1: Change in the bacterial count (log cfu/ml) of treated and untreated zobo drink
A= Untreated, B = treated with 0.5% extract, C = treated with 1% extract, D = 1%sodium benzoate treated. *TNTC = Too numerous to count.

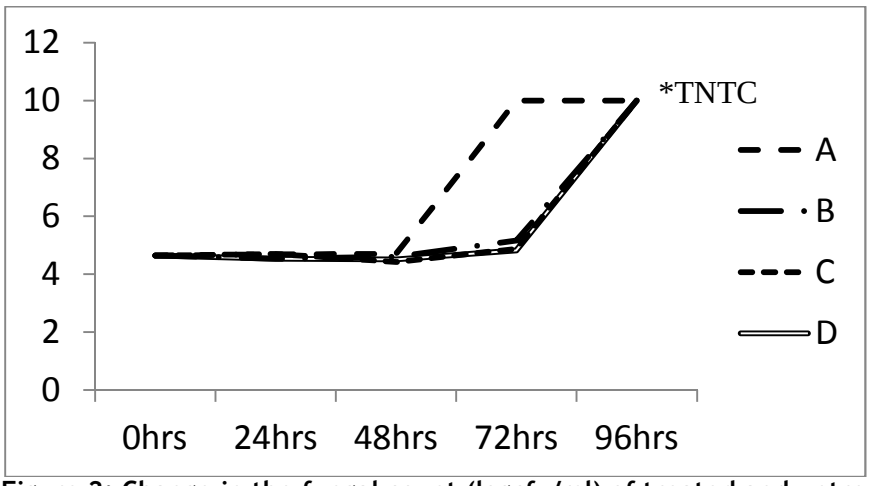


Figure 2: Change in the fungal count (logcfu/ml) of treated and untreated zobo drink
A= Untreated, B = treated with 0.5% extract, C = treated with 1% extract, D = 1%sodium benzoate treated. *TNTC = Too numerous to count.

Bio preservative activity of the extracts on zobo drink showed that the untreated zobo (A) took 24 hours to spoil. This is in line with report by Fasoyiro *et al*. (2005*)* that zobo drink prepared and kept without preservatives have a shelf-life of 24-48 hours. Treatments C and D, however preserved the drink for 48hrs with acceptability score of 5.2(40% acceptability) and 5.4(60% acceptability) respectively.

Table 9: The mean of sensory scores (overall acceptability) of treated and untreated zobo drinks at different storage interval

	Treatments			
Storage(hour)	A	B	C	D
0	8.6±0.24(100)	8.4±0.24(100)	5.6±1.47(100)	5.8±1.49(100)
24	8.4±0.24(100)	8.0±0.32(100)	6.8±0.37(100)	6.8±0.37(100)
48	3.6±0.40(0)	4.8±0.589(40)	5.2±0.37(40)	5.4±0.40(60)
72	1.6±0.24(0)	2.8±0.2(0)	3.2±0.58(0)	3.2±0.37(0)
96	1.0±0.00(0)	2.4±0.40(0)	3.4±0.40(0)	2.8±0.37(0)

Key; A= Untreated zobo, B= zobo treated with 0.5% acetone fraction , C= 1% Acetone fraction, D=1% Sodium benzoate .. The figures enclosed in bracket represent the percentage likeness.
Scale: 9= like extremely, 8= like very much , 7= like moderately, 6= like slightly, 5= neither like nor dislike, 4= dislike slightly, 3= dislike moderately, 2= dislike very much and 1= dislike extremely .

CONCLUSION

The results of the present study have shown the potentials *A. leiocarpus* aqueous and ethanol extracts to revealed the presence of phytochemical such as Saponins, Anthraquinones, Alkaloids, Tannins and Flavonoids in extracts. *Shigella* isolates, values obtained were 20.5±2.5mm, 13.5±0.5mm 12.5±0.05mm and 10.0±0.0mm, respectively through use of ethanol extract. Lastly the extracts have little effect on foods preservation. Conclusively *A .leiocarpus* aqueous and ethanol extracts provide evidence that the plants can be use in preservation of *zobo* drink when the needs arise.

RECOMMENDATION

Further research is therefore recommended to isolate, purify and characterize these chemical constituents with a view to supplementing conventional drugs, sanitizers/preservatives development especially in developing countries.

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